

PATENT COOPERATION TREATY
PCT
INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P3059 WO ORD	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/GB 03/00999	International filing date (day/month/year) 11.03.2003	Priority date (day/month/year) 13.03.2002
International Patent Classification (IPC) or both national classification and IPC A61L27/38		
<p>Applicant UNIVERSITY OF NOTTINGHAM et al.</p>		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 6 sheets, including this cover sheet.
 - This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 21 sheets.
3. This report contains indications relating to the following items:
 - I Basis of the opinion
 - II Priority
 - III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
 - IV Lack of unity of invention
 - V Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
 - VI Certain documents cited
 - VII Certain defects in the international application
 - VIII Certain observations on the international application

Date of submission of the demand 22.08.2003	Date of completion of this report 21.06.2004
Name and mailing address of the international preliminary examining authority:  European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized Officer Menidjel, R Telephone No. +31 70 340-3680



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB 03/00999

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, Pages

- 1-3, 5, 7, 9-13, 16-19, 22-27, as originally filed
30-41, 44, 46, 48
4, 6, 8, 14, 15, 20, 21, 28, 29, received on 28.05.2004 with letter of 28.05.2004
42, 43, 45, 47, 49-51

Claims, Numbers

- 1-20 received on 28.05.2004 with letter of 28.05.2004

Drawings, Sheets

- 1/11-11/11 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
 the language of publication of the international application (under Rule 48.3(b)).
 the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

 contained in the international application in written form.
 filed together with the international application in computer readable form.
 furnished subsequently to this Authority in written form.
 furnished subsequently to this Authority in computer readable form.
 The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
 The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

 the description, pages:
 the claims, Nos.:
 the drawings, sheets:

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5. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	1-20
	No: Claims	
Inventive step (IS)	Yes: Claims	1-20
	No: Claims	
Industrial applicability (IA)	Yes: Claims	1-20
	No: Claims	

2. Citations and explanations

see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

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Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1 - The amendments filed by the applicant **introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT:**

Present claim 2 is an added subject-matter which found no basis within the content of the application as filed. Moreover, the subject-matter of claim 2 can not disclaim the content of the document D1 which is from the same technical field.

2 - The following documents (D1,D2,D3) are referred to in this communication (Article 33(6) PCT); the numbering will be adhered to in the rest of the procedure:

D1: WO 91 09079 A (ERBA CARLO SPA) 27 June 1991 (1991-06-27) cited in the application

D2: HILE D D ET AL: 'Active growth factor delivery from poly(d,l-lactide-co-glycolide) foams prepared in supercritical CO₂' JOURNAL OF CONTROLLED RELEASE, ELSEVIER SCIENCE PUBLISHERS B.V., AMSTERDAM, NL, vol. 66, no. 2-3, May 2000 (2000-05), pages 177-185, XP004193066 ISSN: 0168-3659

D3: WO 02 00275 A (GRIFFITHS IAN ;SEARGEANT KENNETH MALCOLM (GB); VICTREX MFG LTD (GB) 3 January 2002 (2002-01-03)

4. Novelty (Article 33(2) PCT)

- The subject-matter of present claims 1-20 is new for the following reasons (Article 33(2) PCT):

- Document D1 refers to a process for the preparation of a polymer composite wherein active compounds such as therapeutic agents, growth factors are loaded into a polymer directly in one step by using a supercritical fluid technique (Cf. D1, page 8, lines 2-7; page 9, lines 12-19; examples 1-4; claims 1-7).

- Document D2 describes a process for the preparation of a polymer composite, wherein growth factors are loaded into a polymer directly in one step by using a supercritical fluid technique (Cf. D2, the whole document).

- Document D3 refers to a process for the preparation of a polymer foam, wherein active compounds are loaded into a polymer directly in one step using a supercritical fluid technique (Cf. D3, page 1, lines 3-22; page 20, lines 13-33; page 29, line 5-page 30, line 16).

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None of the documents D1-D3 refers to a process as described in present claim 1, wherein the preparation of a polymer composite is loaded with functioning matter selected from mammalian, plant and bacterial subcellular, cellular or multicellular matter, liposomes, and aggregates and mixtures thereof, wherein the process comprises contacting a polymer substrate and functioning matter with a plasticising fluid which is capable of plasticising polymer in its natural state or in supercritical, near critical, dense-phase or subcritical state having fluid density in the range 0.001 g/ml up to 10 g/ml under plasticising conditions.

5. Inventive Step (Article 33(1),(3) PCT)

- The subject-matter of present claims 1-20 is considered as being inventive for the following reasons (Article 33(1),(3) PCT):
 - The subjective problem of the present application is to provide a method for loading functioning matter selected from mammalian, plant and bacterial subcellular, cellular or multicellular matter, liposomes and aggregates and mixtures thereof, in one step, in contrast with forming a scaffold and seeding with said functioning matter (see application, on page 4, lines 13-16).
 - Document D1, which is considered as the most relevant state of the art, describes a process for the preparation of a polymer composite wherein **active compounds such as therapeutic agents, growth factors are loaded into a polymer directly in one step by using a supercritical fluid technique** (Cf. D1, page 8, lines 2-7; page 9, lines 12-19; examples 1-4; claims 1-7).
 - The objective technical problem may be seen as to provide a process for forming cell-laden scaffolds in one step.
 - The solution provided by the present application is a process for the preparation of a polymer composite loaded with mammalian, plant and bacterial subcellular, cellular or multicellular matter, liposomes, and aggregates and mixtures thereof using a process as described in present claim 1.
 - The difference between the claimed subject-matter and the teaching of the closest prior art is the load within the polymer matrix of functioning matter selected from mammalian, plant and bacterial subcellular, cellular or multicellular matter, liposomes, and aggregates and mixtures thereof with a plasticising fluid which is capable of plasticising polymer in its natural state or in supercritical, near critical, dense-phase or subcritical state having fluid density in the range 0.001 g/ml up to 10 g/ml under plasticising conditions

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- Neither D1-D3 nor their combination renders the subject-matter of present claims 1-20 obvious. Therefore, the subject-matter of present claims 1-20 involves an inventive step (Article 33(1),(3) PCT).

6. Industrial Application (Article 33(4) PCT)

- The subject-matter of present claims 1-20 is considered to be industrially applicable; claims 1-20 therefore, satisfy the criterion set forth in Article 33(4) PCT.

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Polymers are also being developed in biomedical applications as biological cell-laden scaffolds for use as biomedical inserts such as bone inserts, and as organ and tissue modules for *in vitro* and *in vivo* use, as inserts or for *in vivo* studies. A considerable effort is being invested in developing scaffolds which encourage

5 growth and development of particular types of living matter and in particular configurations to mimic living systems. Cell-loading is typically by dropping a cellular soup onto the scaffold surface and allowing to permeate in whereby cells are seeded and are found to grow and proliferate into and throughout the scaffold itself on culturing. This is relatively time consuming, the rate limiting

10 factor being the cell growth and proliferation, moreover each scaffold must be configured for the desired growth configuration.

It is an object of the present invention to provide a method for loading cells into polymers directly, i.e. for instant production of cell-laden scaffolds, in one step,

15 in contrast with forming a scaffold and seeding with cells. To date no one has found a means to achieve this, and keeping the cells alive.

“Bacterial inactivation by using near- and supercritical carbon dioxide” Dillow *et al*, Proc. Natl. Acad. Sci. USA, Vol 96 pp 10344-10348, Aug 1999 discloses

20 contacting bacterial cells with SC CO₂ under supercritical conditions of 25 – 35C, 2.05 x 10⁷ Nm² (205 bar) in the absence and presence of polymer microspheres. Contact times of 0.1 to 4 hours, typically 30 and 45 minutes were employed. Contacting was reported to achieve bacterial inactivation and thus sterilisation of polymer where present. Sterilisation and virus inactivation kits

25 using SCF are available.

We have now surprisingly found, contrary to the indications of Dillow, that the properties of plasticising fluids in general may be employed in the preparation

ART 34 AMENDMENT

- for the preparation of a polymer composite loaded with functioning matter wherein the process comprises contacting a polymer substrate and an amount of functioning matter with a plasticising fluid, or mixture of plasticising fluids, under plasticising conditions for a period sufficient to plasticise and/or swell
- 5 the polymer and incorporate the functioning matter, and releasing the plasticising fluid to obtain the polymer composite, wherein contacting is at a pressure in the range $1 \times 10^5 \text{ Nm}^{-2}$ to $1 \times 10^8 \text{ Nm}^{-2}$ (1 to 1000 bar) and a temperature in the range -200 to +500°C selected in manner that at least a proportion of functioning matter does not freeze or refreeze during processing,
- 10 or if at a temperature at which freezing or refreezing may occur, that either matter is desiccated or a pressure constraint is applied, whereby pressure is in a range having a maximum of less than $1 \times 10^8 \text{ Nm}^{-2}$ (1000 bar) throughout contact of functioning matter and plasticising fluid, whereby at least a proportion of functioning matter retains its function in the polymer composite.
- 15 For example we have observed that freezing or refreezing may take place during processing with carbon dioxide as plasticising fluid at least in the temperature range +4 to +35°C.

- Reference herein to functioning matter is to matter which under favourable
- 20 conditions is capable of performing a function, such as growth, movement, metabolism of substances, generation of substances for example, or other functioning typically attributed with living matter and whose function is reversibly or irreversibly prejudiced under non favorable conditions. Functioning matter according to the invention may not be actively functioning
- 25 at all times but may be dormant or inactive at any given time, and in particular is unlikely to be actively functioning during the process of the invention. Functioning matter may be chemically or physically preserved prior to contact

ART 34 ARD'T

- composite, wherein contacting is with cryopreserved or non cryopreserved functioning matter and is conducted at a temperature in a range at which functioning matter remains frozen or unfrozen, or thaws without refreezing during contacting, for example -100 to +4 °C, +35 to +100°C or is with
- 5 cryopreserved or non cryopreserved functioning matter and is conducted at a temperature at least in the range +4 to +35°C and maximum pressure less than $1 \times 10^8 \text{ Nm}^{-2}$ (1000 bar), which may be less than $4 \times 10^7 \text{ Nm}^{-2}$ (400 bar) or $2.75 \times 10^7 \text{ Nm}^{-2}$ (275 bar), for example in the range $5 \times 10^5 \text{ Nm}^{-2}$ to $75 \times 10^5 \text{ Nm}^{-2}$ (5 to 75 bar) throughout contact of functioning matter and plasticising
- 10 fluid, whereby functioning matter retains its function in the polymer composite.

- Reference herein to functioning matter is to matter capable of performing or exhibiting specific functioning, and whose functioning is harmed by contact with plasticising fluid under plasticising conventional conditions. For example
- 15 SCF dissolves or diffuses into biological cells and like enclosed fluid matter such as liposomes with harmful effect on cell functioning or liposome content functioning. Biological cells comprise liquid contents and SC CO₂ can easily penetrate, permeate and cause damage to these contents.
- 20 Chemical or physical preservation according to the invention preferably comprises rendering functioning matter dormant, and therefore is reversible. Preferably preservation comprises rendering functioning matter in plasticising fluid-impenetrable state and/or isolating functioning matter contents which facilitate plasticising fluid damage.
- 25 Preferably functioning matter is non cryopreserved, ie non frozen or is physically preserved by cryopreservation, i.e. freezing, preferably at a temperature in the range -10 to -100°C, most preferably - 20 to - 75°C.

ART 3A AMENDT

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(CO₂).

Optimum temperature in these ranges may depend in part on the nature of functioning matter to be processed and on the plasticising fluid or fluids to be employed, as hereinbefore described. For most fluids a typical temperature may

- 5 be in the range approximately 10 to 15°C, 15 to 25°C, 25 to 30°C, 30 to 35°C, 35 to 45°C or 45 to 55°C. Other sub ranges may be envisaged and are within the scope of the invention. Preferably the lowest temperature is employed which is compatible with sufficient lowering of the polymer Tg to achieve plasticisation. To operate at ambient temperature, the process of the invention may require
10 compensation by increase in pressure.

Throughout contact with plasticising fluid, under conditions which typically comprise ambient temperature or elevated temperature, frozen functioning matter is preferably maintained frozen, for example by conducting the process
15 in a vessel and cooling the vessel; or is allowed to thaw during processing, which also maintains viability. Significantly the process of the invention ensures frozen functioning matter is not allowed to thaw and refreeze during processing which can prejudice viability.

- Plasticising fluid typically comprises a pressure less than, equal to or greater
20 than the plasticising fluids critical pressure (P_c) from in excess of 1 x 10⁵ Nm⁻² to 1 x 10⁹ Nm⁻² (1 bar to 10000 bar), preferably 1 x 10⁵ Nm⁻² to 1 x 10⁸ Nm⁻² for example 2 x 10⁵ Nm⁻² to 8 x 10⁷ Nm⁻² (1 to 1000 bar, for example 2 to 800 bar). Preferably plasticising conditions according to the present invention
25 comprise a pressure in the range 2 x 10⁵ Nm⁻² to 4 x 10⁷ Nm⁻² (2 to 400 bar), more preferably 5 x 10⁵ Nm⁻² to 2.65 x 10⁷ Nm⁻² (5 to 265 bar), most preferably 5 x 10⁵ Nm⁻² to 75 x 10⁵ Nm⁻² (5 to 75 bar). In the case of functioning matter preserved by imposing pressure constraint we have surprisingly found that operation at a

~~ART 3A AMENDT~~

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pressure in a preferred range of $5 \times 10^5 \text{ Nm}^{-2}$ to $75 \times 10^5 \text{ Nm}^{-2}$ (5 to 75 bar) and at temperatures in the range of +4 to +37 °C maintains viability of functioning matter.

- 5 Optimum pressure in these ranges may depend in part on the nature of functioning matter to be processed and on the plasticising fluid or fluids to be employed, as hereinbefore described. For most fluids pressure will be in the range approximately $3 \times 10^6 \text{ Nm}^{-2}$ to $4 \times 10^6 \text{ Nm}^{-2}$ (30 to 40 bar), $4 \times 10^6 \text{ Nm}^{-2}$ to $5 \times 10^6 \text{ Nm}^{-2}$ (40 to 50 bar), $5 \times 10^6 \text{ Nm}^{-2}$ to $6 \times 10^6 \text{ Nm}^{-2}$ (50 to 60 bar),
10 $6 \times 10^6 \text{ Nm}^{-2}$ to $7.5 \times 10^6 \text{ Nm}^{-2}$ (60 to 75 bar), most preferably approximately $3.4 \times 10^6 \text{ Nm}^{-2}$ to $7.5 \times 10^6 \text{ Nm}^{-2}$ (34 to 75 bar) (dense phase or supercritical CO₂). Other sub ranges may be envisaged and are within the scope of this invention.

Fluid may be provided at plasticising conditions prior to contacting with
15 polymer and functioning matter or may be brought to plasticising conditions in contact with one or both of polymer and functioning matter. Swelling and plasticisation may be simultaneous or sequential or plasticisation may occur without swelling.

The process is conducted for a suitable contact time of plasticising fluid and
20 functioning matter which can be employed without prejudicing functioning of matter. For example it is important that the process is conducted with contact time such that there is little or no thawing of frozen functioning matter. In a particular advantage the process may be carried out for very short contact time of plasticising fluid and functioning matter of 2 milliseconds up to 10 minutes,
25 more preferably 20 milliseconds to 5 minutes, more preferably 1 second to 1 minute, more preferably 2 to 30 seconds, most preferably 2 to 15 seconds. In this case a non-uniform distribution may be acceptable. Alternatively contact

~~ART 54(A)2007~~

- be subjected to conditions of elevated temperature and pressure increasing density thereof up to and beyond a critical point at which the equilibrium line between liquid and vapour regions disappears. Supercritical fluids are characterised by properties which are both gas like and liquid like. In particular,
- 5 the fluid density and solubility properties resemble those of liquids, whilst the viscosity, surface tension and fluid diffusion rate in any medium resemble those of a gas, giving gas like penetration of the medium

Preferred plasticising fluids include carbon dioxide, di-nitrogen oxide, carbon disulphide, aliphatic C₂-10 hydrocarbons such as ethane, propane, butane, pentane, hexane, ethylene, and halogenated derivatives thereof such as for example carbon tetrafluoride or chloride and carbon monochloride trifluoride, and fluoroform or chloroform, C₆-10 aromatics such as benzene, toluene and xylene, C₁-3 alcohols such as methanol and ethanol, sulphur halides such as sulphur hexafluoride, ammonia, xenon, krypton and the like, or a mixture thereof. Typically these fluids may be brought into plasticising conditions at temperature of between -200°C to + 500°C and pressures of in excess of 1 x 10⁵ Nm⁻² to 1 x 10⁹ Nm⁻² (1 bar to 10000 bar), as hereinbefore defined. It will be appreciated that the choice of fluid may be made according to its properties, for example diffusion and polymer plasticisation. Preferably the fluid acts as solvent for residual components of a polymer composite as hereinbefore defined but not for polymer or functioning matter as hereinbefore defined. Choice of fluid may also be made with regard to critical conditions which facilitate the commercial preparation of the polymer as hereinbefore defined. Supercritical conditions of some fluids are shown in Table 1.

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Table 1

Fluid	Critical Temperature / °C	Critical Pressure / $\times 10^5$ Nm $^{-2}$ (bar)
Carbon dioxide	31.1	73.8
Ethane	32.4	48.1
Ethylene	9.3	49.7
Nitrous oxide	36.6	71.4
Xenon	16.7	57.6
Fluoroform CHF ₃	26.3	48.0
Monofluoromethane	42	55.3
Tetrafluoroethane	55	40.6
Sulphur hexafluoride	45.7	37.1
Chlorofluoromethane	29	38.2
Chlorotrifluoromethane	28.9	38.7
Nitrogen	-147	33.9
Ammonia	132.5	111.3
Cyclohexane	280.3	40.2
Benzene	289.0	48.3
Toluene	318.6	40.6
Trichlorofluoromethane	198.1	43.5
Propane	96.7	41.9
Propylene	91.9	45.6
Isopropanol	235.2	47.0

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and bioceramics, other minerals, hyaluronic acid, polyethyleneoxide, CMC (carboxymethylcellulose), proteins, organic polymers, and the like and components adapted for incorporation as implants into meniscus, cartilage, tissue and the like and preferably promote growth, modelling, enhancing or 5 reinforcing of collagen, fibroblasts and other natural components of these host structures.

Additional biofunctional component(s) may be mixed with the polymer and functioning matter or may be introduced by subsequent soaking or impregnation of functioning matter laden product composite.

10

Biofunctional components may be present in any desired amount for example as hereinbefore defined for functioning matter. For example a composite may comprise 80 wt% hydroxyapatite, 10 wt% cells, less than 1 wt% growth factor and more than 1 wt% antibiotic.

15

Accordingly in a preferred embodiment there is provided according to the present invention a process as hereinbefore defined for the preparation of a polymer composite comprising biofunctional matter as hereinbefore defined and loaded with functioning matter as hereinbefore defined wherein the process

20 comprises in a first stage contacting the biofunctional material and polymer and a plasticising fluid as hereinbefore defined under plasticising conditions as hereinbefore defined to plasticise and/or swell the polymer and incorporate the biofunctional material and subsequently introducing an amount of functioning matter and combining with polymer, and releasing the plasticising fluid to 25 obtain the polymer composite, wherein contacting is at a pressure in the range $1 \times 10^5 \text{ Nm}^{-2}$ to $1 \times 10^8 \text{ Nm}^{-2}$ (1 to 1000 bar) and a temperature in the range

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-200 to +200°C selected in manner that at least an amount of functioning matter does not freeze or refreeze during processing or if at a temperature at which freezing or refreezing may occur, that either matter is desiccated or a pressure constraint is applied in a range having a maximum pressure less than 1 x 10⁸

- 5 Nm⁻² (1000 bar) throughout contact of functioning matter and plasticising fluid, whereby at least an amount of functioning matter retains its function in the polymer composite. Or example we have observed that freezing or refreezing may take place during processing with carbon dioxide as plasticising fluid at least in the temperature range +4 to +35°C. Functioning matter may be
10 chemically or physically preserved against harmful effects associated with the plasticising fluid as hereinbefore defined and is maintained in preserved state preferably in dry form, for example may be cryopreserved or desiccated throughout contact therewith. Alternatively matter may be in hydrated, non frozen form.

15.

- If porous, a composite may comprise open or closed cell pores. Composite obtained with a very open porous structure, known as microcellular, is ideal for biomedical and biocatalytic applications for example supporting growth of blood vessels and collagen fibres throughout the matrix, and forming structures
20 resembling bone, meniscus, cartilage, tissue and the like, and providing a structure for throughput of substrate for biocatalysis and bioremediation and the like.

- Non-porous, open or closed cell composite may be useful for biodegradable
25 staged or prolonged release delivery applications of functioning matter or biofunctional material not requiring leaching in or out or other access. Release may be *in-vitro* or *in-vivo* and by parenteral, oral, intravenous, application or

Figure 4 shows scanning electron micrograph images of cell-laden polymer composite fabricated according to the invention; viability of murine 3T3 fibroblasts is observed, with post processing incubation from Days 1 to 8, as described in the Examples showing spreading and attachment of cells and plaque formation

Figure 5 shows a scanning electron micrograph image of the cell-laden polymer composite of Figure 4 at Day 8 in greater magnification.

Figure 6 shows the viability of Ovine meniscal fibrochondrocytes (OMC) and 3T3 Fibroblasts after exposure to high pressure CO₂ for increasing time periods. Frozen cells were exposed to high pressure CO₂ at 68 x 10⁵ Nm⁻² (68 bar) and a temperature of 4°C for time periods of 1 to 30 minutes. Cells were then cultured and the viability of each cell type was measured.

Figure 7 shows the effect of temperature on Ovine meniscal fibrochondrocytes (OMC) and 3T3 Fibroblasts. Frozen cells were exposed to high pressure CO₂ at 68 x 10⁵ Nm⁻² (68 bar), for an exposure time of 66 seconds at starting exposure temperatures from 4 to 60°C.

Figure 8 shows the effect of pressure on frozen Ovine meniscal fibrochondrocytes (OMC) and 3T3 Fibroblasts. Frozen cells were exposed to high pressure CO₂ between 34 and 204 x 10⁵ Nm⁻² (34 and 204 bar), the starting exposure temperature remained constant at 4°C and the exposure time was fixed at 66 seconds..

Figure 9 shows proliferation of primary chondrocytes over an 8 day period after exposure to a range of pressures of CO₂. Cells had been frozen and exposed to high pressure CO₂ between 34 and 204 x 10⁵ Nm⁻² (34 and 204 bar), at a starting exposure temperature of 4°C and exposure time of 66 seconds.

Figure 10 shows the effect of temperature on Ovine meniscal fibrochondrocytes (OMC). In this example cells were not frozen prior to exposure to high pressure CO₂ at 68 x 10⁵ Nm⁻² (68 bar), for an exposure time of 66 second, at starting exposure temperatures from 4 to 60°C.

Figure 11 shows the effect of pressure on Ovine meniscal fibrochondrocytes (OMC). These cells were not frozen prior to exposure to high pressure CO₂ between 34 and 204 x 10⁵ Nm⁻² (34 and 204 bar);; the starting exposure temperature remained constant at 4°C and the exposure time was fixed at 66 seconds.

Materials and Methods

Isolation of Cells

Ovine meniscal fibrochondrocytes (OMC) were isolated from the stifle joint of 3-6 month old lambs (slaughtered for the food chain) and the fibrochondrocytes isolated by digestion with pronase E (0.01 g/g cartilage; Merck, UK) for 3 hours 5 at 37°C at 5%CO₂, followed by further digestion with collagenase (type II 0.02 g/g of cartilage; Lorne Laboratories, UK) for 18 hours at 37°C 5% CO₂. Cells were passed through a 70 µm filter (to remove undigested tissue), washed by centrifugation and expanded in culture in a humidified atmosphere at 37°C, 5% CO₂ in DMEM supplemented with 10% FCS, 10% (v/v) foetal calf serum 10 (FCS), 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and

Pressurisation of CO₂

For all experiments, pressurised CO₂ was pumped through the system by a
5 Pickel pump (PM-1013; New Wave Analysis, Switzerland), through a back
pressure regulator (model number BP-1580-81; Jasco, UK), to a 10 ml Thar cell
autoclave (Thar Designs Inc, USA). For length of exposure experiments, the
Thar cell was filled with high pressure CO₂ at a constant rate of 2.83 x 10⁵ Nm⁻²
10 (2.83 bar/s) until 68 x 10⁵ Nm⁻² (68 bar) was reached. The vessel was held
at pressure for the desired length of time (1, 3, 5, 15, and 30 min) and the vessel
vented until the pressure had returned to atmospheric (total time for filling and
venting 37.5 s). For effect of pressure and temperature experiments, the
autoclave was filled to desired pressure in 24 s (rate 2.83 x 10⁵ Nm⁻²
15 (2.83bar/s)), this pressure was held for 12 s, and the autoclave vented for 30 s
(total time 66 s). Temperature was controlled using ice (4°C), cold water
(15°C), room temperature (22°C), and a heating block (specially designed to
hold the autoclave) for 37°C, 45°C, and 60°C.

Conditions for frozen cells

20 For all experiments using frozen cells, cells were kept frozen in liquid N₂
vapour prior to exposure to high pressure CO₂. Following exposure, cells were
stored on ice prior to being transferred to a 24 well tissue culture plastic plate
with 1 ml warm complete media. Cells were incubated at 37°C, 5% CO₂ for 3
hours to allow for cell adhesion. The media was carefully removed so as to not
25 disturb the cells and replaced with fresh (1 ml). Cells were incubated for a
further 21 hours (day 1) prior to Alamar Blue assay. For cells cultured for more
than one day, the Alamar Blue was removed at the end of the assay and replaced

Example 1 – Isolation of Functioning matter

Biological cells were prepared as either frozen or desiccated particles. A suspension of mouse 3T3 fibroblasts in 10% DMSO in FCS (foetal calf serum) containing 0.1M HEPES buffer pH 7 was frozen in a mould at -70°C for at least 2 hours (and up to one week) prior to exposure to dense phase CO₂. The resulting cell pellet was immersed in liquid nitrogen and then physically disrupted with a pestle and mortar to form particles with average diameters of between 50 and 1000 micron. DMSO acts as a cryopreservation agent allowing freezing and thawing without damage, as known in the art.

Example 2a – Composite formation of Polymer with homogeneously dispersed isolated Functioning matter, by dense phase fluid processing within the autoclave.

Starting materials were poly (DL-lactic acid) (100 – 200 mg) and frozen mammalian cells obtained from Example 1. The frozen cells were seeded 5 between two part-formed PLA slices and these were placed within an autoclave. The temperature was maintained at 4°C. Carbon dioxide was pumped into the autoclave until a pressure of $68 \times 10^5 \text{ Nm}^{-2}$ (1000 psi, 68.03 bar) was achieved. A stirrer within the autoclave was rotated to combine the polymer and cell particles. In this case the stirrer was a helical impeller. After 10 seconds of 10 exposure the pressure was rapidly released by opening a valve. The composite comprising polymer loaded with frozen cells was then removed from the autoclave and stored frozen in liquid nitrogen.

Example 2b

Alamar blue assay, using a scaffold processed in the same way but without cells as the assay blank. Following the Alamar blue assay, scaffolds were washed with PBS and a small piece cut and fixed in 3% glutaraldehyde at 4°C for at least overnight prior to further processing for SEM. The remaining scaffold was cultured further in complete medium and the above repeated at day 4 and 8.

Example 6 – Viability of frozen functioning matter following exposure to high pressure CO₂

10 Ovine meniscal fibrochondrocytes (OMC) or 3T3 fibroblasts suspended in a modified freezing medium were frozen at -80°C overnight in a mould. These solid frozen cell pellets, each containing 2×10^5 cells/ml, were exposed to high pressure liquid CO₂ (4°C and 68×10^5 Nm⁻² (68 bar)) for increasing time periods and cultured for 24 hours in complete culture medium. The viability of 15 each cell type measured using the Alamar Blue assay and quoted as a percentage of control cells is shown in Figure 6. Populations of both cell types retained over 30% viability after 1 min of exposure. After 3 min exposure only the OMC population retained viability. After 5 min exposure both populations had died.

20 Example 7 – Effect of Temperature on frozen functioning matter

OMC or 3T3 fibroblasts were exposed to high pressure liquid CO₂ according to the method of Example 6. The pressure was kept constant at 68×10^5 Nm⁻² (68 bar), the total exposure time was fixed at 66 seconds and the starting exposure temperature was varied. The cells were then cultured as described in 25 Example 6 and the viability of each cell type measured using the Alamar Blue assay and quoted as a percentage of control cells. The results in Figure 7 show

a trend of increased population viability, for both cell types, at higher temperatures. At 45 and 60°C the population viability approaches 100% of the control. Heating occurs as CO₂ is pumped rapidly into the vessel and results in a partial or complete thawing of the cell pellet at all starting temperatures.

- 5 Then, as the pressure falls rapidly at the end of the exposure period a sharp fall in temperature may occur. It appears that at starting temperatures of 4, 15 and 22°C the Joules-Thompson cooling effect is sufficient to re-freeze the cell pellet with the expected loss of cell viability resulting from rapid ice crystal formation. In contrast at 37, 45 and 60°C the autoclave is partially insulated against the
10 final temperature fall and the cells remain within the recently thawed suspension.

Example 8 Pressure range for frozen functioning matter

OMC or 3T3 fibroblasts were exposed to high pressure liquid CO₂ according to the method of Example 6. The total exposure time and starting exposure temperature were fixed. The results shown in Figure 8 show cell viability after exposure to pressures between 34 x 10⁵ Nm⁻² and 204 x 10⁵ Nm⁻² (34 and 204 bar). For all experiments we observed variability in the cell survival data resulting from the effect of cryopreservation on overall viability of both cell type populations. However, accepting the inherent noise within the data from cryopreserved cells, there is a drop of population viability from 70% at 34 x 10⁵ Nm⁻² (34 bar) to less than 50% at greater pressures. The effect of Joules-Thompson heating and cooling complicates data interpretation as temperature, pressure and exposure time cannot be independently varied. However, the observation of cell survival up to 204 x 10⁵ Nm⁻² (204 bar) remains valid. A further step in the analysis of the primary chondrocytes post CO₂ exposure is

shown in Figure 9 where cell proliferation over an 8 day period is evident. Similar rises in cell number were recorded after exposure to all pressures.

Example 9 – Viability of non-frozen functioning matter following exposure to high pressure CO₂

The cell suspension, in modified freezing medium, was diluted to the desired cell number and transferred to the moulds as described previously. Cells were kept on ice prior to and following exposure to high pressure CO₂ conditions and then transferred to complete cell culture media for culture. Cell viability was assessed using the Alamar Blue viability assay as described in Example 5. Figure 10 shows the effect of temperature on the viability of non-frozen OMC. At temperature of 12, 22 and 37°C over 60% viability was maintained but at higher and lower temperatures viability decreased to less than 40%. Figure 11 shows the effect of pressure on the viability of non-frozen OMC. At 4°C, OMC population survival was unaffected by short exposure to 34 x 10⁵ Nm⁻² (34 bar) pressure of CO₂ but at pressures of 68 bar and above viability was compromised.

Further aspects and advantages of the invention will be apparent from the foregoing.

CLAIMS

1. A process for the preparation of a polymer composite loaded with functioning matter directly in one step, wherein functioning matter is selected from mammalian, plant and bacterial subcellular, cellular or multicellular matter, liposomes, and aggregates and mixtures thereof wherein the process comprises contacting a polymer substrate and functioning matter with a plasticising fluid or mixture of plasticising fluids comprising a liquid or gaseous fluid which is capable of plasticising polymer in its natural state or in supercritical, near critical, dense-phase or subcritical state having fluid density in the range 0.001 g/ml up to 10 g/ml under plasticising conditions to plasticise and/or swell the polymer and incorporate the functioning matter, and releasing the plasticising fluid to obtain the polymer composite, wherein contacting is for a short contact time of 20 milliseconds up to 5 minutes at a pressure in the range 1×10^5 to $1 \times 10^8 \text{ Nm}^{-2}$ (1 to 1000 bar) and a temperature in the range -200 to +500C, selected in manner that at least a proportion of functioning matter does not freeze or refreeze during processing, or if at a temperature at which freezing or refreezing may occur, that either matter is desiccated or a pressure constraint is applied whereby pressure is in a range having a maximum pressure less than $1 \times 10^8 \text{ Nm}^{-2}$ (1000 bar) throughout contact of functioning matter and plasticising fluid, whereby at least a proportion of functioning matter retains its function in the polymer composite.
- 25 2. A process as claimed in Claim 1 with the proviso that it is carried out for very short contact time of plasticising fluid and functioning matter of less than 3 minutes.

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3. A process as claimed in any of Claims 1 and 2 wherein contacting is with cryopreserved or non cryopreserved functioning matter and is conducted at a temperature at least in the range +4 to +35°C and maximum pressure less than $4 \times 10^7 \text{ Nm}^{-2}$ (400 bar) throughout contact of functioning matter and plasticising fluid.
4. A process as claimed in any of Claims 1 to 3 contacting is with cryopreserved or non cryopreserved functioning matter and is conducted at a temperature at least in the range +4 to +35°C and maximum pressure less than 10 $2.75 \times 10^7 \text{ Nm}^{-2}$ (275 bar), for example in the range $5 \times 10^5 \text{ Nm}^{-2}$ to $75 \times 10^5 \text{ Nm}^{-2}$ (5 to 75 bar).
5. A process as claimed in any of Claims 1 to 4 wherein contacting is with carbon dioxide as plasticising fluid wherein freezing or refreezing may take 15 place during processing at least in the temperature range +4 to +35°C, and a pressure constraint is applied.
6. A process as claimed in any of Claims 1 to 5 wherein at least 20% of functioning matter maintains function.
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7. A process as claimed in any of Claims 1 to 6 wherein plasticising conditions comprise a pressure in the range $2 \times 10^5 \text{ Nm}^{-2}$ to $4 \times 10^7 \text{ Nm}^{-2}$ (2 to 400 bar), more preferably $5 \times 10^5 \text{ Nm}^{-2}$ to $2.65 \times 10^7 \text{ Nm}^{-2}$ (5 to 265 bar).
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8. A process as claimed in any of Claims 1 to 7 wherein plasticising fluid includes carbon dioxide, di-nitrogen oxide, carbon disulphide, aliphatic C₂₋₁₀ hydrocarbons such as ethane, propane, butane, pentane, hexane, ethylene, and halogenated derivatives thereof such as for example carbon tetrafluoride or chloride and carbon monochloride trifluoride, and fluoroform or chloroform, 30 C₆₋₁₀ aromatics such as benzene, toluene and xylene, C₁₋₃ alcohols such as

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methanol and ethanol, sulphur halides such as sulphur hexafluoride, ammonia, xenon, krypton, or a mixture thereof.

9. A process as claimed in any of Claims 1 to 8 wherein functioning matter is present in an amount with respect to polymer of 1×10^{-12} wt% to 99.9 wt%.

10. A process as claimed in any of Claims 1 to 9 wherein functioning matter is selected from mammalian, plant and bacterial cells including (subcellular) organelles and aggregates thereto including pancreatic islet or liver spheroids and the like; and liposomes as carrier of sensitive matter.

11. A process as claimed in any of Claims 1 to 10 wherein functioning matter is selected from mammalian and plant prokaryotic and eukaryotic cells and mixtures and aggregates thereof; and liposomes as carrier of protein or enzymes.

12. A process as claimed in any of Claims 1 to 11 wherein functioning matter comprises mammalian cells selected from fibroblasts, fibrochondrocytes, chondrocytes, bone forming cells such as osteoblasts and osteoclasts, bone marrow cells, hepatocytes, cardiomyocytes, blood vessel forming cells, neurons, myoblasts, macrophages, microvascular endothelium cells and mixtures thereof and collagen, and liposomes.

25 13. A process as claimed in any of Claims 1 to 12 for the preparation of a polymer composite additionally comprising biofunctional material and loaded with functioning matter wherein the process comprises in a first stage contacting biofunctional material selected from drugs and veterinary products, agrochemicals, human and animal health products, human and animal growth promoting, structural or cosmetic products, and absorbent materials for

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poisons and toxins and polymer and a plasticising fluid or a mixture of plasticising fluids under plasticising conditions to plasticise and/or swell the polymer and incorporate the biofunctional material.

- 5 14. Process as claimed in any of Claims 1 to 13 wherein polymer is selected from: polyesters including poly(lactic acid), poly(glycolic acid), copolymers of lactic and glycolic acid, copolymers of lactic and glycolic acid with poly(ethylene glycol), poly(*e*-caprolactone), poly(3-hydroxybutyrate), poly(p-dioxanone), poly(propylene fumarate); poly (ortho esters);
10 polyanhydrides; Poly(amino acids); polyacetals; polyketals; polyorthoesters; Polyphosphazenes; azo polymers; synthetic Non-biodegradable Polymers selected from: Vinyl polymers including polyethylene, poly(ethylene-co-vinyl acetate), polypropylene, poly(vinyl chloride), poly(vinyl acetate), poly(vinyl alcohol) and copolymers of vinyl alcohol and vinyl acetate, poly(acrylic acid)
15 poly(methacrylic acid), polyacrylamides, polymethacrylamides, polyacrylates, Poly(ethylene glycol), Poly(dimethyl siloxane), Polyurethanes, Polycarbonates, Polystyrene and derivatives; and Natural Polymers selected from carbohydrates, polypeptides and proteins.
- 20 15. A polymer composite comprising a polymer loaded with functioning matter selected from mammalian, plant and bacterial subcellular, cellular or multicellular matter and aggregates and mixtures thereof obtainable by the process as defined in any of Claims 1 to 14, wherein at least 20% of functioning matter has retained function in the polymer composite.
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16. A polymer composite comprising a polymer loaded with functioning matter selected from mammalian, plant and bacterial subcellular, cellular or multicellular matter and aggregates and mixtures thereof as defined in any of Claims 1 to 14 wherein functioning matter is non-established, ie is directly loaded functioning matter, and is not proliferated, grown, adhered or otherwise
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modified post loading, at least 20% of which has retained function in the polymer composite.

17. A polymer composite as claimed in Claim 15 or 16 which is in granular
5 or monolith form.

18. A polymer composite loaded with functioning matter selected from mammalian, plant and bacterial subcellular, cellular or multicellular matter and aggregates and mixtures thereof as claimed in any of Claims 15 to 17,
10 additionally comprising biofunctional materials, selected from drugs and veterinary products, agrochemicals, human and animal health products, human and animal growth promoting, structural or cosmetic products, and absorbent materials for poisons and toxins, suitably sized and shaped for a desired application.

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19. A polymer composite, a scaffold thereof or the process for the preparation thereof as claimed in any of Claims 1 to 18 for use as a support or scaffold for drug delivery, for use in bioremediation, as a biocatalyst or biobarrier for human or animal or plant matter, for use as a structural component, for example comprising the polymer and optional additional synthetic or natural metal, plastic, carbon or glass fibre mesh, scrim, rod or like reinforcing for medical or surgical insertion, for insertion as a solid monolith into bone or tissue, as fillers or cements for wet insertion into bone or teeth or as solid aggregates or monoliths for orthopaedic implants such as pins, or dental implants such as crowns etc.

20. A process for preparing a polymer composite, a polymer composite, a scaffold, or the use thereof substantially as described in the description or illustrated in the Examples.

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